IMPAIRED OPSONIZATION OF PNEUMOCOCCUS IN PATIENTS WITH SICKLE CELL ANEMIA

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IMPAIRED OPSONIZATION OF PNEUMOCOCCUS IN PATIENTS WITH SICKLE CELL ANEMIA

by

Robert E. Galloway

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DEDICATION

To Mom and Dad

for everything.

ACKNOWLEDGEMENTS

My profound appreciation is extended to Dr. Oswaldo Castro and Dr. Howard A. Pearson to whom I owe more than I can express for their inspiration, support, advice and assistance not only with this study, but with many other aspects of my medical school career. A special thanks to Mrs. Young Soo Park for technical assistance.

INTRODUCTION

Increased susceptibility to bacterial infections in patients with Hemoglobin SS has been well documented. Although earlier studies emphasized salmonella infections, recent investigators have shown that pneumococcus is the most frequent offender[3]. The exact pathogenesis of this susceptibility is not established. However serum factors which promote the phagocytosis of pneumococcus, i. e. opsonins, have been reported to be deficient in children with sickle cell anemia, according to a test system utilizing leukocyte-HBG suspensions and stored serum[29]. Also, children with sickle cell anemia have been reported to have functional asplenia[20], and the role of the spleen in early antibody response to antigenic stimulation is apparently impaired in this patient population[25]. The purpose of this investigation is twofold: (1) to study serum opsonic capacity in patients with sickle cell anemia in a more physiologic test system using a simple whole blood phagocytic-bactericidal test for pneumococcus, and (2) to determine whether or not the growth of pneumococcus is different in Hgb SS versus Hgb AA blood.

METHODS AND MATERIALS

A. Patient Group

A total of fourteen (14) patients, 3 to 27 years of age (average of 12 years old) were studied. Twelve (12) of these had homozygous hemoglobin S disease (Hgb SS). Only two had palpable spleens; they ranged from 4 to 6 years of age. The others, 8 through 20 years of age, were presumably asplenic as a result of autoinfarction[8]. Seven (7) of the 12 patients with homozygous Hgb S were female. The other two in the study included 2 males, 5 and 27 years of age with SC disease (Hgb SC). The nature of the study was explained in detail and informed parental consent was obtained in accordance with the provisions set forth in the Declaration of Helsinki.

The following was obtained from all patients and recorded: (a) hematocrit, (b) morphology, (c) white blood cell count, (d) differential, (e) hemoglobin electrophoresis, (f) hemoglobin F, (g) hemoglobin A, and (h) percent S. None of the patients had been transfused or on any antibiotics within the past month. Only patient #11 required hospitalization to rule out pneumococcal sepsis. All of the others were seen on an outpatient basis.

TABLE I.

Subjects with Sickle Cell Hemoglobin States

Patient	Int.	Age	Sex	Hct.	Type	F	A_2	%S	WBCx10^3	Seg:Band
1	J.J.	5	M	26.0	SC	1.6	2.1	47.2	11.5	30:3
2	S.B.	20	F	20.0	SS	12.0	2.6	85.4	9.2	43:1
3	J.W.	15	F	28.0	SS	1.9	3.6	94.5	13.1	71:1
4	P.W.	6	M	34.0	SS	10.1	3.3	86.6	4.9	49:1
5	A.G.	8	F	33.0	SS	15.7	1.9	82.4	9.9	37:1
6	S.E.	19	F	21.0	SS	5.6	2.3	92.1	6.5	46:1
7	A.P.	27	M	18.0	SC	2.8	1.7	40.0	10.4	43:5
8	B.C.	10	M	15.0	SS	9.0	7.0	84.0	14.8	48:1
9	A.E.	15	F	24.0	SS	2.0	3.2	94.8	15.7	48:10
10	D.G.	19	F	17.0	SS	12.4	1.3	86.3	14.1	55:13
11	K.Z.	6	F	25.0	SS	6.0	3.0	91.0	15.0	44:8
12	M.M.	12	F	18.0	SS	6.4	3.8	89.8	9.3	50:1
13	T.L.	11	M	13.9	SS	4.3	2.7	93.0	12.8	41:5
14	J.W.	15	M	16.0	SS	1.9	3.6	95.5	6.4	52:3

* Seg:Band refers to the ratio of mature to immature neutrophills.

The control subjects included 6 healthy volunteers. All disavowed having had any medications within one month prior to the time the blood samples were taken and all were hemoglobin AA_2 type. Hemoglobin F levels were also normal (< 1.0%). Of the 6 controls (5 adults and 1 child) 3 were black. As with the patient group, age and sex were recorded with all pertinent data (Table II).

TABLE II.

Volunteers for Controls

Subject	Int.	Age	Sex	Hct.	Type	F	A_2	$WBCx10^3$	Seg:Band
1	O.C.	41	M	44	AA_2	0.6	2.6	8.1	54:1
2	R.G.	29	M	45	AA_2	0.6	2.5	6.3	56:0
3	M.M.	21	F	43	AA_2	0.7	3.2	5.5	59:5
4	E.M.	35	F	44	AA_2	0.9	2.8	4.1	67:0
5	C.K.	23	F	43	AA_2	0.7	2.9	6.1	70:3
6	M.A.	8	M	41	AA_2	0.8	2.4	8.9	72:3

B. Type, Source, and Maintenance of Pneumococcus

The best culture media for pneumococcus was found to be a fresh beef infusion broth containing 10% serum or blood, and titrated to a final pH of 7.4 to 7.8. A reducing agent,

i.e. cysteine, is added to prevent oxidation; and for storing the organism, catalase was added as red blood cells (RBC's) to promote decomposition of H_2O_2 and thereby insure viability. A simulated counterpart of this methodology is described by Holt[13], and consists of the following:

TABLE III.

1. Basal Medium:

NaCl	2.5 gms
KH_2PO_4	0.5 gms
$MgSO_4 \cdot 7H_2O$	0.4 gms
$CaCl_2$ (dried)	1.0 ml 1% ($^W/_V$) soln.
Proteose peotone (Difco) ..	20.0 gms
L-Cysteine HCl	0.15 gms
Yeast extract (Difco)	5.0 gms
Distilled H_2O	1000.0 cc
Adjusted to	pH 7.2

2. 50% ($^W/_V$) glucose in distilled water.

3. 20% ($^W/_V$) Na_2CO_3 solution.

A qualitative estimate was made of the growth rates of the type 3 pneumococcus from stock cultures in: (a) serum, (b) beef broth, glucose, carbonate, and 0.2% ($^{V}/_{V}$) Holt's stock solution, and (3) beef broth, glucose, carbonate and 0.1% ($^{V}/_{V}$) blood. Since serum is expensive and contaminates the microbes with antigenic material, an efficient substitute was welcomed. Colony counts were determined by pour plate technique with blood agar (although chocolate agar gave a slight more luxurient growth, colonies were easier visualized in the lighter blood agar plates). Relative counts were recorded in Table IV and suggested that blood was superior with respect to growth enhancement. Media (c) and blood agar pour plates (pour 30 cc melted trypticase soy agar into space saver place containing 1 cc bank blood* plus specific aliquot of specimen) were used throughout the experiment[27].

TABLE IV

Pneumococcus Type III

18 Hours at 34° on Pour Plates

		Count (million/ml)
(a)	Control (serum)	600
(b)	Beef broth, Glucose, Carbonate & Holt's .	1500
(c)	Beef broth, Glucose, Carbonate & Blood ..	2900

*Blood stored at 4° C for > 1 week in acid, citrate and dextrose solution (ACD) is devoid of leukocytic activity.

The type 8 pneumococci was isolated from the blood of subject #11 and typed by the quellung reaction at the Yale University Hospital laboratories. The organisms for the phagocytic tests were prepared by placing 5 drops of the stock (2.5 cc of infected blood and 5.0 cc of defibrinated guinea pig blood) in 7.5 ml of media (c). Two dozen test tubes of media and stock were incubated at 37°C for 6 hours when turbidity was maximal (previously determined maximum turbidity from trials with type III). Bacteria were counted in a Petroff-Hauser chamber, so that 1.0 ml contained 2.0×10^8 bacteria. Immediately, 1 ml aliquots of the turbid broth were pipetted into 3 ml screw-top, pyrex test tubes #3790 containing 0.05 ml of glycerol as a preservative. All tubes were immediately sealed and quick frozen (first at -20°C in acetone and dry ice, then at maintenance of -70°C in an automatically controlled, preset refrigerator unit). This maneuver eliminated the need to maintain viability (via mouse passage) and insured uniform purity and numbers of culture specimens.

C. Preparation of the Experimental Procedure

1. Resistance to osmotic shock.

Since the phagocytic-bactericidal experiment used osmotic shocks to lyse leukocytes, the effect of this

procedure on pneumococcus was studied. One ml of quick frozen pneumococcal specimen was spontaneously thawed at 37° C. A 10^{-5} dilution of bacterium was made with serum, Holt's special medium and bank blood, into respective 25 ml siliconized Erlenmeyer flasks. The 3 flasks were maintained in an automatic agitated water bath at 37° C. Melted trypticase soy agar was maintained in a special water bath at 57° C, in tubes of 15 ml capacity. Pour plates were made every 30 minutes of two 0.1 ml aliquots of the flask contents, one directly and one after elution into 10 mls of isotonic saline for 1 minute, i.e. 6 pour plates per 30 minutes. By placing bacterial specimens in this water media one could measure the effect, if any, of osmotic shock (lysis) upon the organism secondary to the isotonic solution.

2. Standardization deoxygenation apparatus.

A special compressed gas mixture (5% CO_2, 4.5% O_2, and 90.5% N_2) stored in a leakproof aluminum tank was allowed to flow via adaptor and leakproof rubber and glass tubing (interconnected) into several 25 ml Erlenmeyer flasks in series. Tank pressure was 1500 lbs/sq. inch and flow was gently bubbled through an initial flask of distilled water -- thus insuring adequate humidification of samples. All rubber-stopper sealed flask units had (in addition to the glass tubing) a central spinal needle

(gauge #18) with stylet -- the tip of which remained beneath the fluid surfaces. This particular modification allowed periodic sampling of blood suspensions in order to determine partial pressures of 0_2 and $C0_2$; removal of specific aliquots for pour plating and incubation; and microscopic viewing of the morphological alterations induced by various gaseous tensions and mixtures. A 10^{-5} dilution of bacterium in bank blood was made x 4 (in 25 ml Erlenmeyer flasks) and equilibrated to 37° C in an agitated water bath. Two flasks were in series with the gas flow and therefore exposed to low $p0_2$ and low $pC0_2$. Two other flasks were exposed to room 0_2 via an open neck straight glass tubing. A final flask was similar to the latter two in all respects, except it contained 4.5 ml serum (normal*) as opposed to bank blood -- this sample served as a control. Blood gases were taken and measured periodically to insure patency of the system (see Figure IA).

3. Phagocytic-bactericidal assay.

Fourty (40) ml of venous blood were obtained from several volunteers under asceptic conditions. These blood samples were processed immediately after determining blood grouping. Serum samples were tested within one hour.

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*Serum was taken from healthy laboratory workers.

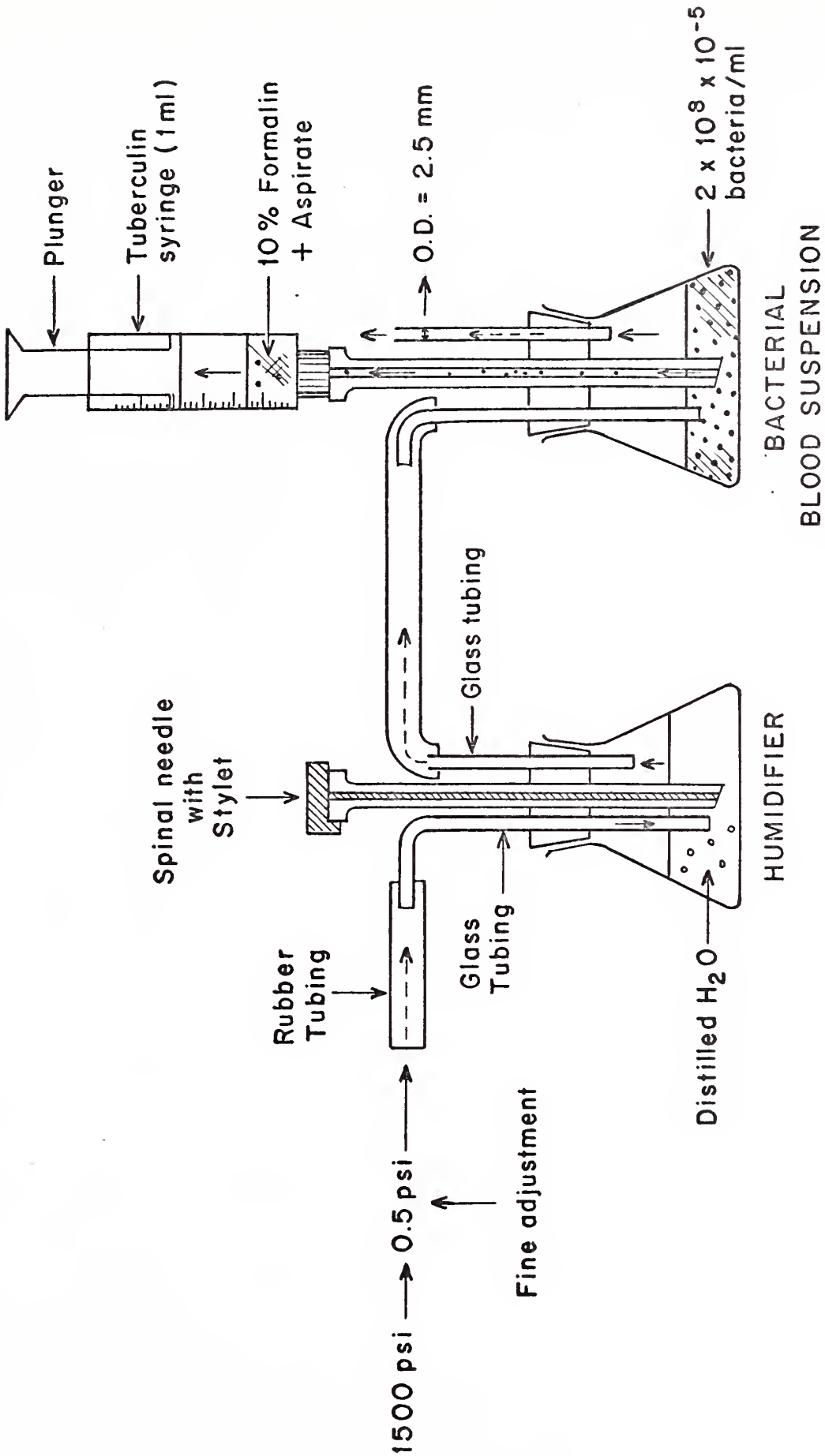


Figure 1A: SCHEMA OF DEOXYGENATION SYSTEM. A gas mixture of 5% CO_2, 4.5% O_2, and 90.5% N_2 flows gently through a series of flasks interconnected via adaptors, glass and rubber tubing. Humidifier and sample flasks are maintained at 37° C in a water bath shaker.

Simultaneous with a white cell count determination, each 40 ml aliquot was pipetted into a sterile 50 cc Erlenmeyer flask containing 20 glass beads (1 x 1 mm) and defibrinated for 10 minutes. Four 4.5 ml aliquots of the defibrinated blood were placed in sterile siliconized 25 ml Erlenmeyer flasks in the agitated water bath. Two (2) were assembled to the deoxygenator in series, and 2 were exposed to room air via open neck glass tubing with an external diameter of 2.5 mm. The residual defibrinated blood was centrifuged at 1000 rpm's for 10 minutes. A 4.5 ml aliquot of serum supernatant was then eluted into a fifth 25 ml Erlenmeyer as a control. Next, 0.1 ml aliquots of bacteria, spontaneously thawed at 37° C, were immediately diluted to 10^{-5} organisms/ml in each of the 5 flasks. At 15 minute intervals, 0.1 ml aliquots were pipetted from all samples and plated in duplicate (one directly and one post elution x 1 minute in 10 cc isotonic saline). A final maneuver was employed to accurately measure the extent of intracellular killing by leukocytes[1]. When an antibiotic (200 U/ml of penicillin) was added to the test suspension, e.g. 0.1 ml of bacterial media in 10 cc sterile isotonic saline, only bacteria associated with white blood cells were counted from samples collected at 30 minutes from zero time of phagocytosis.

Cells collected by centrifugation were washed three times. The error of osmotic disruption of the leukocytes were insignificant since bacterial/neutrophill ratios were low[5]. All samples, test and control, were then plated according to the aforementioned pour plate technique.

RESULTS

I. Effect of Osmotic Shock, 0_2 Variation, and Sickle Blood Substitution Upon Growth Characteristics of Pneumococcus.

A. The Growth Curve.

Figure 1B shows the growth curve of the pneumococcus strain used. After a lag phase of 2 hours, the number of bacteria increased approximately 2 logs over 4-5 hours, remained stationary for 15 hours, and began to decrease after 20 hours total. The phagocytic-bactericidal assays (see Figure 1B) to study the changes due to leukocyte activity were subsequently carried out within the first 2 hours of the growth phase in a constant bacterial population.

B. Effect of Osmotic Shock.

A trial experiment was conducted for 5 hours with diluted and nondiluted specimens. Post plating and over-

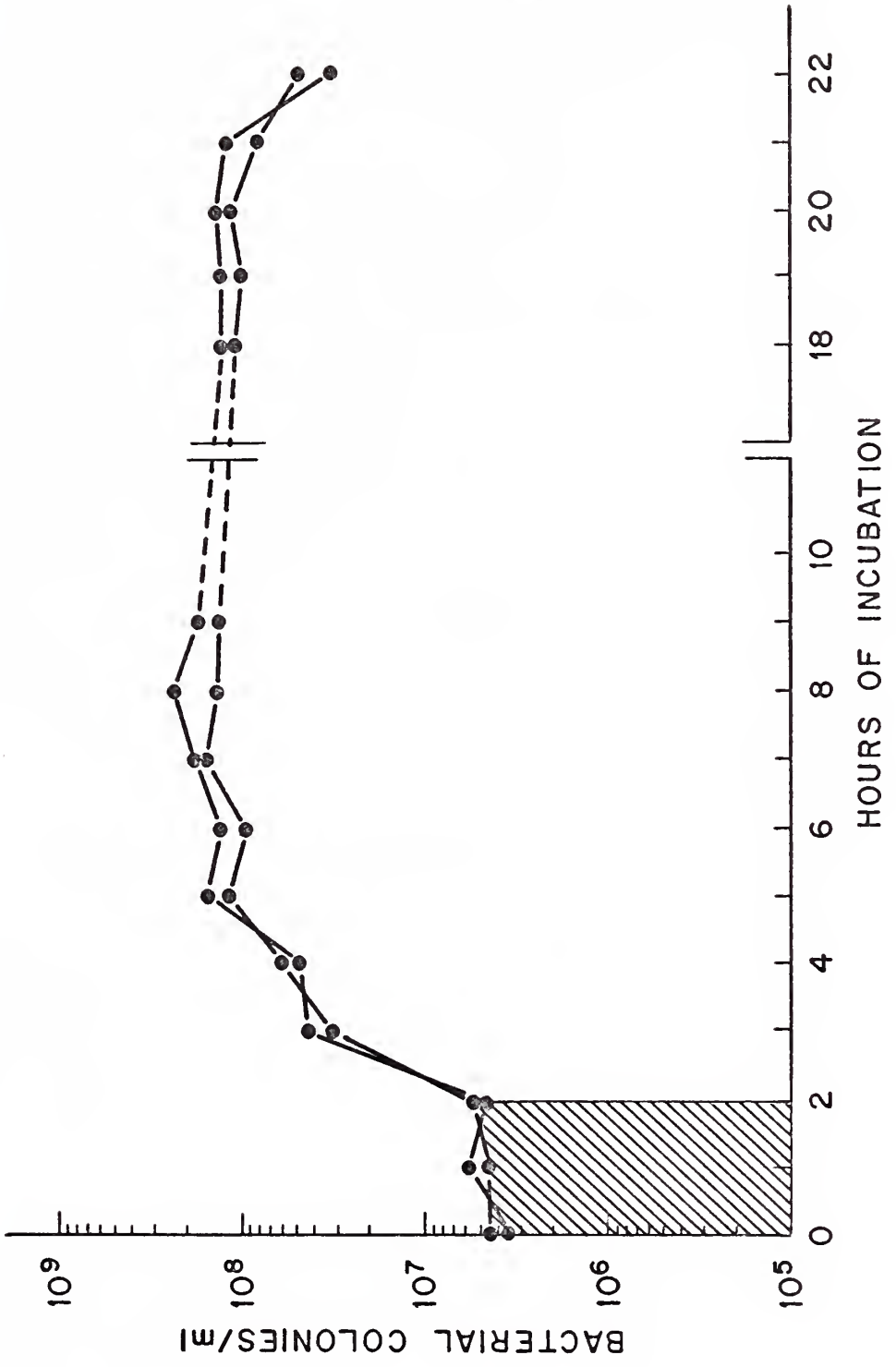


Figure 1B: Growth curve of the type III pneumococcus strain used. After a lag phase of 2 hours the number of bacteria increased by approximately 2 logs over the next 4-5 hours. The growth then remained stationary for 15 hours.

night incubation, the colonies were counted (see Figure II).

Figure II shows that there were no appreciable differences between the colony counts of the samples treated with and without osmotic shock. The effect of osmotic shock on bacteria appeared minimal regardless of whether bacterial suspensions were in serum, Holt's media or blood. Although all curves are relatively stable during the initial 2 hours, the system supported by blood appeared to have a slight enhanced growth rate.

C. Effect of Oxygen Tension on the Growth of Pneumococcus.

Samples, both in (low 0_2) and out (high 0_2) of series, were obtained each hour x 5. Blood gases were obtained (see Figure III) and proved the system closed, leakproof, and functioning. The figure shows that the deoxygenation apparatus produced severe hypoxia in the blood-bacteria suspension. There was, however, no difference in the growth curve of pneumococcus between the samples tested at room air versus low 0_2.

D. Growth of Pneumococcus in Sickle Cell Blood (Devoid of Leukocytes) With and Without Hypoxia.

A 40 ml blood sample was obtained under sterile technique from patient #2 and stored at 4° C for 7 days in a siliconized flask with ACD solution to eliminate

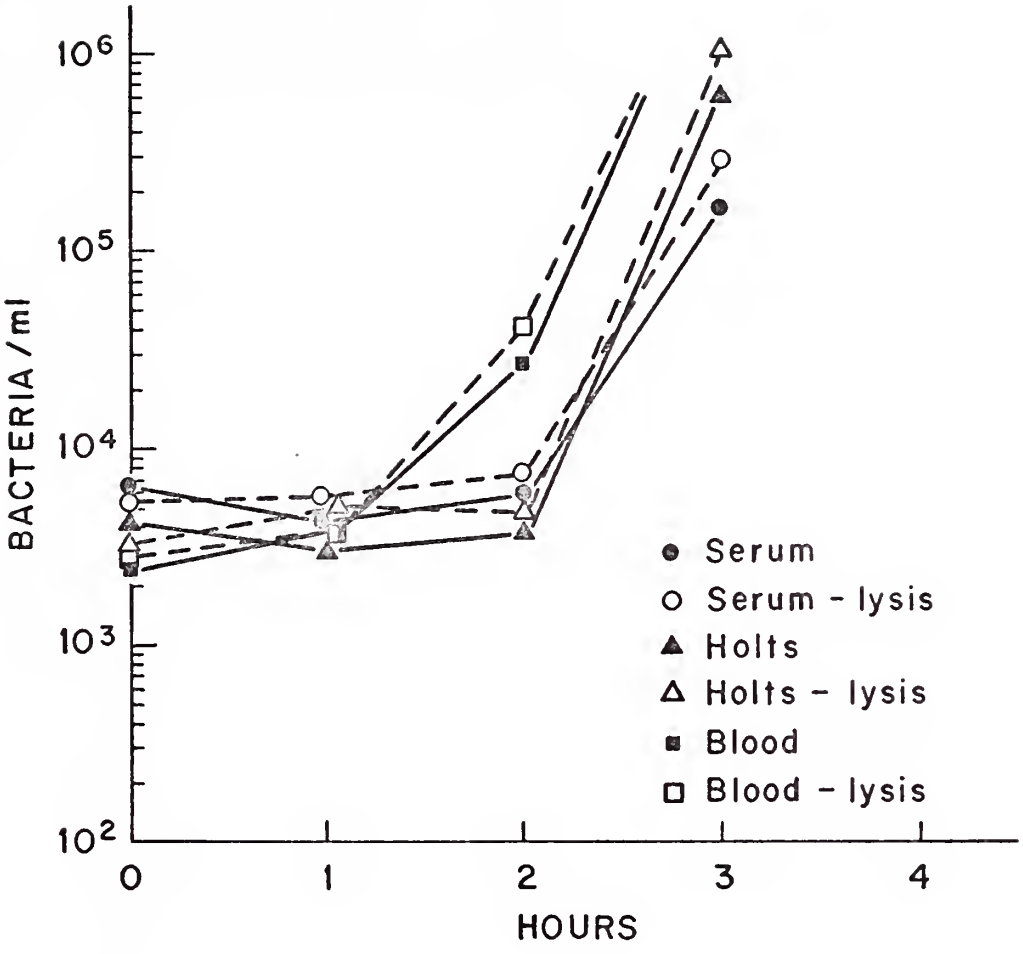


Figure II: Effect of osmotic shock upon the growth of pneumococcus in serum, Holt's media, and bank blood. No significant difference noted regardless of whether samples diluted (----) or non-diluted (——).

Gases	pH	pCO_2	pO_2	% O_2 Sat.
$\downarrow O_2$	6.74	39	45	44
$\uparrow O_2$	6.82	24	87	83

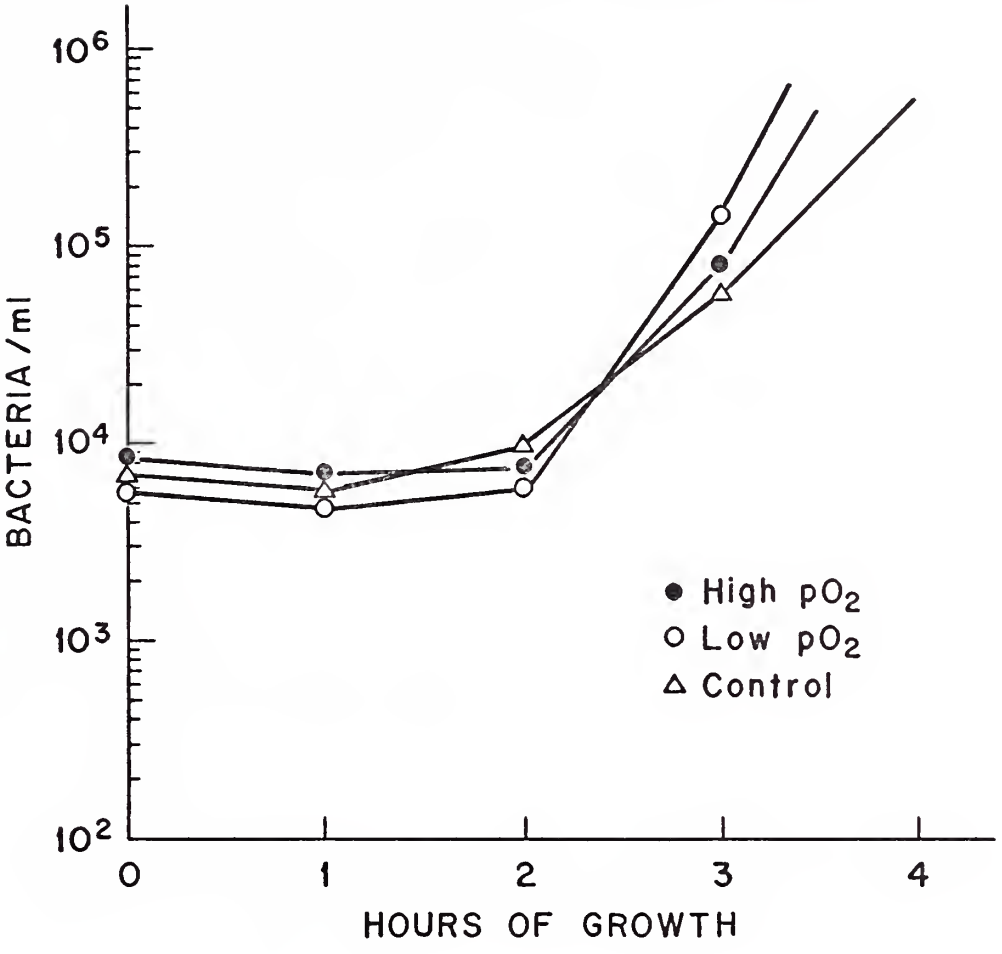


Figure III: Effect of oxygen tension on growth of pneumococcus in bank blood. Blood gases (see insert) confirms hypoxia. No difference in growth was observed regardless of whether samples exposed to hypoxic atmosphere or to room air.

viable leukocytes[27]. The pneumococcal growth curve was the same in this blood as that in control blood regardless of low 0_2 tension. Blood gases were repeated (see Figure IV).

At the second hour from zero time, an aliquot of Hgb SS blood was obtained from each flask in series. Aspiration was into a 1.0 cc tuberculin plastic disposable syringe containing 0.1 cc of formic acid. Red blood corpuscles were immediately fixed, plated beneath glass cover slip and viewed microscopically to access the percentage of crescent cells. A random count of 100 cells in duplicate revealed 84 and 86% sickled RBC's. Only 5-10% of the cellular population was sickled when flasks exposed to room air were similarly prepared. No change was obvious in the curves as related to increased or decreased oxygen tensions. Blood gases were constant.

E. Effect of Whole Blood (Blood Leukocyte Phagocytic-Bactericidal Activity).

Control subjects: Figure V shows the decrease in colony counts in the blood of a normal individual's serum did not have this effect, suggesting that the decreased colony counts is due to leukocyte phagocytic/ bactericidal activities. The colony counts in the lysed samples represent the total number of bacteria present at each sampling trial, i.e. intracellular and extra-

Gases	pH	pCO_2	pO_2	% O_2 Sat.
$\downarrow O_2$	6.79	41	38	44
$\uparrow O_2$	6.80	28	86	81

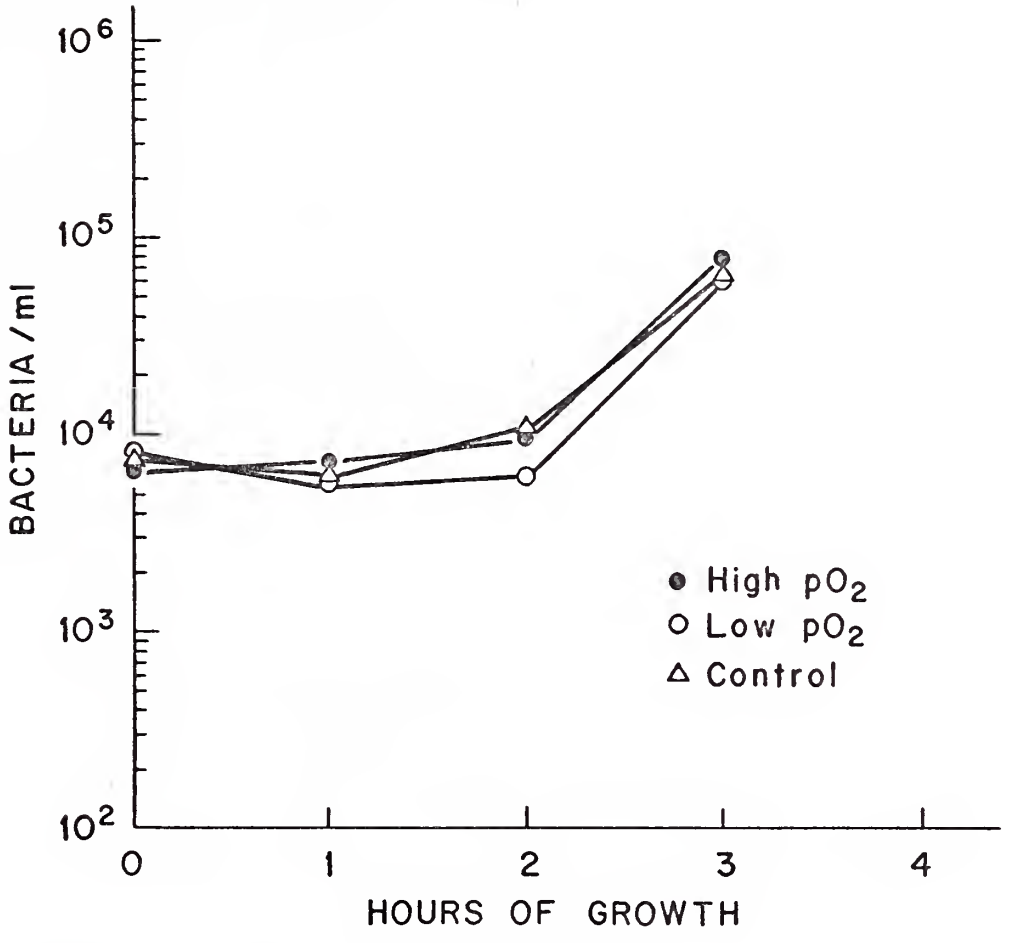


Figure IV: Growth of pneumococcus in sickle cell blood (devoid of leukocytes) with and without hypoxia. No difference noted regardless of whether samples were exposed to hypoxia or room air.

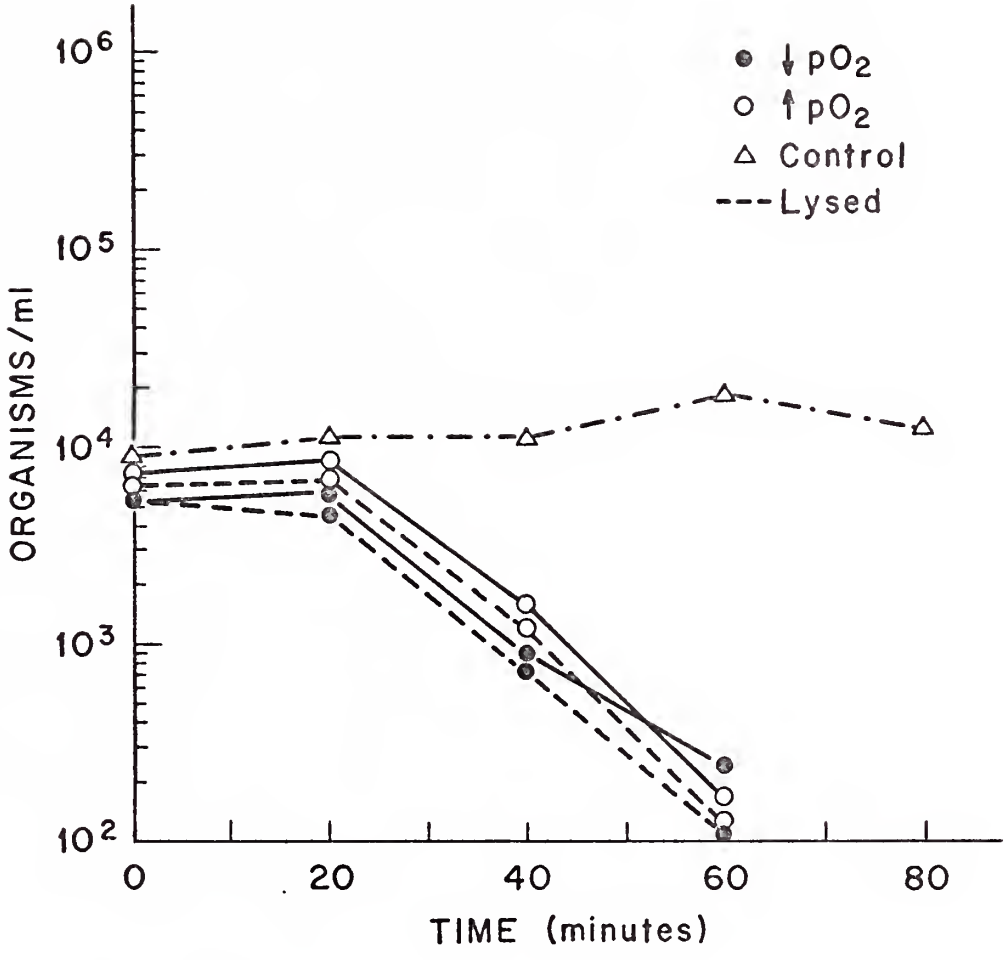


Figure V: Blood leukocyte phagocytic-bactericidal activity of control subjects. While the number of pneumococcus type VIII remained stationary in serum (–·–·–·) both lysed (----) and non-lysed (———) whole blood specimens decreased with comparable rates.

cellular bacteria. The colony counts seen with unlysed samples represent extracellular (unphagocytized) bacteria since the intracellular bacteria were either killed or their growth was inhibited when the leukocytes which had phagocytized them were plated intact. This was proven as follows: A 40 ml aliquot of blood was obtained under asceptic conditions from a clinically normal, electrophectically proved Hgb AA volunteer. Post defibrination, 4.5 ml aliquots of suspensions were eluted into series and nonseries siliconized flasks followed by measured bacterial inoculums. Leukocyte response was observed for 1 hour. There was no significant difference between oxygenated and deoxygenated systems. The WBC count was 6550. Partial pressures of the gas mixtures remained stable (see Figure V[4]).

F. Ingested Versus Nonphagocytized Bacteria.

In order to demonstrate that at each sampling time the overwhelming majority of viable bacteria are extracellular, and that the assay is therefore an accurate reflection of phagocytosis, part D was repeated with the singular modification of adding 200 U/ml of penicillin to the test suspension (0.1 ml of blood-bactericidal suspension in 10 cc of sterile isotonic saline) after 20 minutes. Duplicate aliquots were obtained at 30 minutes

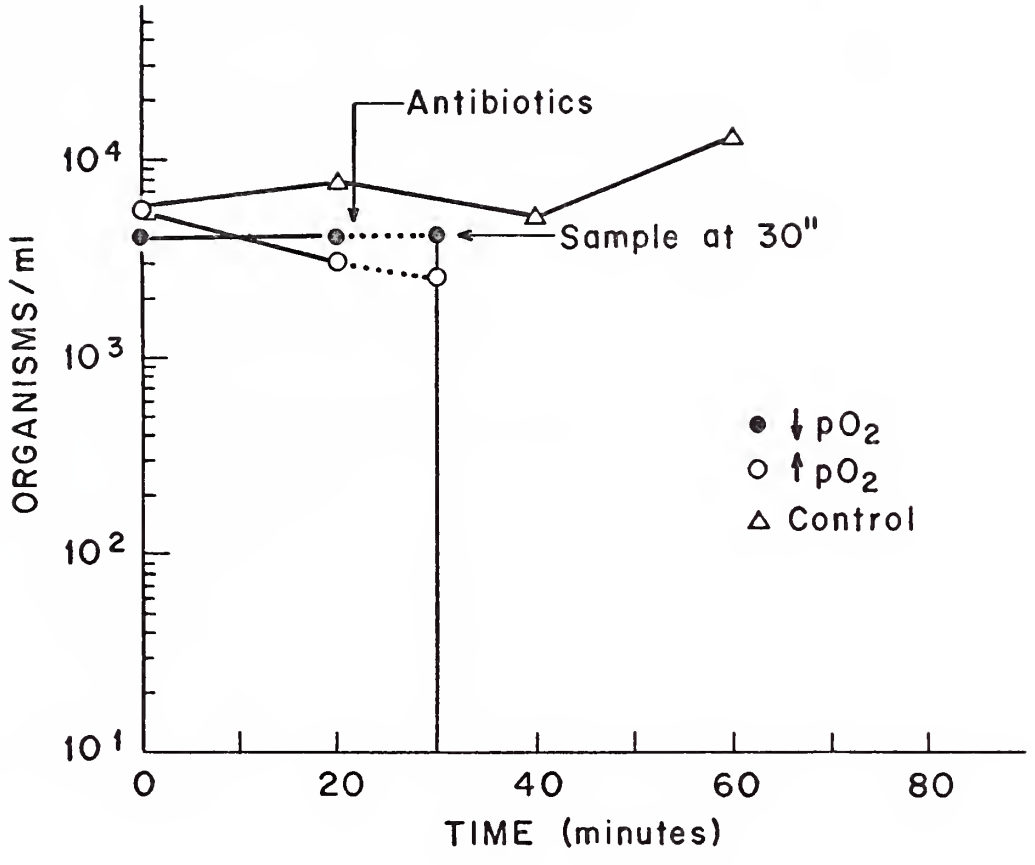


Figure V[1]: Intracellular killing by normal phagocytes. A sample of normal blood was incubated with bacteria for 20 minutes. After this time 200 U/ml of penicillin was added to the test system to kill extracellular organisms. Colony count at 30 minutes was zero indicating intracellular killing of pneumococcus.

from zero time of phagocytosis. Following centrifugation and washing X3, cells were plated according to the pour plate technique. The abrupt decline in the slope at 30 minutes strongly suggested intracellular killing of phagocytized bacteria in that population of leukocytes. Also of note is that the hypoxia had no demonstrable effect on the leukocyte phagocytic/bactericidal activity (see Figure V).

II. Standards of Deviation for Controls and Study Group.

A. Controls.

The phagocytic and bactericidal functions of PMN's for 6 normal (5 adult laboratory workers and 1 child volunteer) people were studied as a control for the systems. Respective rates of phagocytosis were determined and all curves integrated into one graph (see Figure VI) with standards of deviation and percentage of phagocytosis per unit time. The half-life for the summated curve was 12.75 minutes. Blood gases were $p0_2$ = 39 and $pC0_2$ = 39.

B. Patient Group.

1. Table V summarizes the patient population included in the first portion (part A) of the study. Eight (8) patients were chosen for this phase, however,

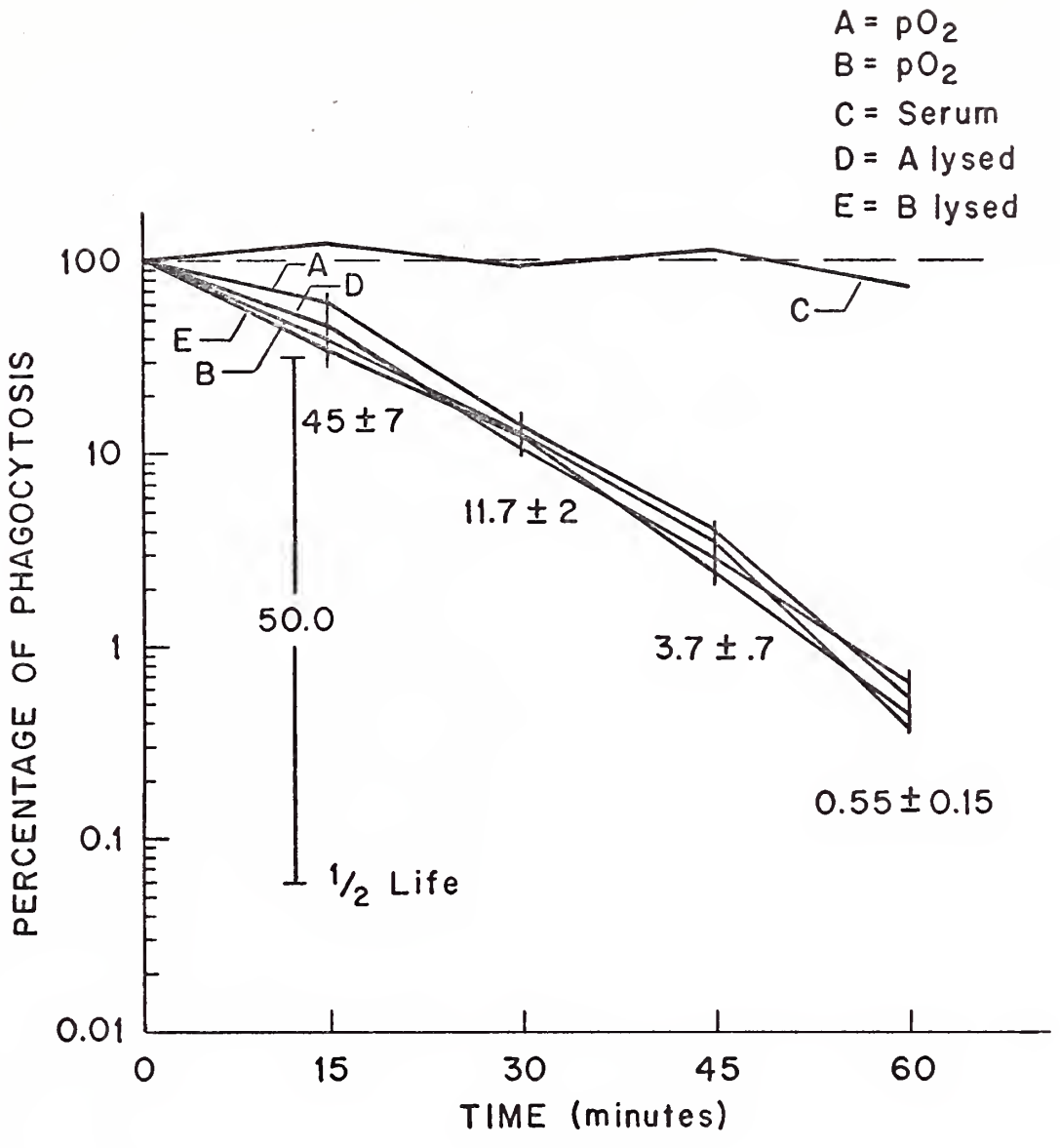


Figure VI: Phagocytic-bactericidal effect of blood from control subjects. Mean and standard deviations for 6 normal individuals determined. The 100% point on the ordinate represents the number of bacteria present at zero time. The half life of viable organisms in the system was 12.75 and the average WBC count is 6.5×10^3. No effect of hypoxia is demonstrated.

two of these* later abandoned the study because of impending symptomatology suggestive of "crises". Part A was performed identically as the control with the exception that bloods of patients with the S hemoglobinopathy were used in lieu of normal blood. Figure VII shows that the pneumococcus in patients' blood (Hgb SS) was similar to that of the control group. Also, no effect of hypoxia could be demonstrated.

TABLE V.

Pt.	Int.	Age	Sex	Hct.	Type	F	A_2	%S	WBCx10^3	Seg:Band
1	J.J.	5	M	26	SC	1.6	2.1	47.2	11.1	30:3
2	S.B.	20	F	20	SS	12.0	2.6	85.4	9.2	43:1
3	J.W.	15	F	28	SS	1.9	3.6	94.5	13.1	71:1
4	P.W.	6	M	34	SS	10.1	3.3	86.6	4.9	49:1
* 5	A.G.	8	F	33	SS	15.7	1.9	82.4	9.9	37:1
6	S.E.	19	F	21	SS	5.6	2.3	92.1	6.5	47:1
7	A.P.	27	M	18	SC	2.8	1.7	40.0	10.4	43:5
* 8	B.C.	10	M	15	SS	9.0	7.0	84.0	14.8	48:1

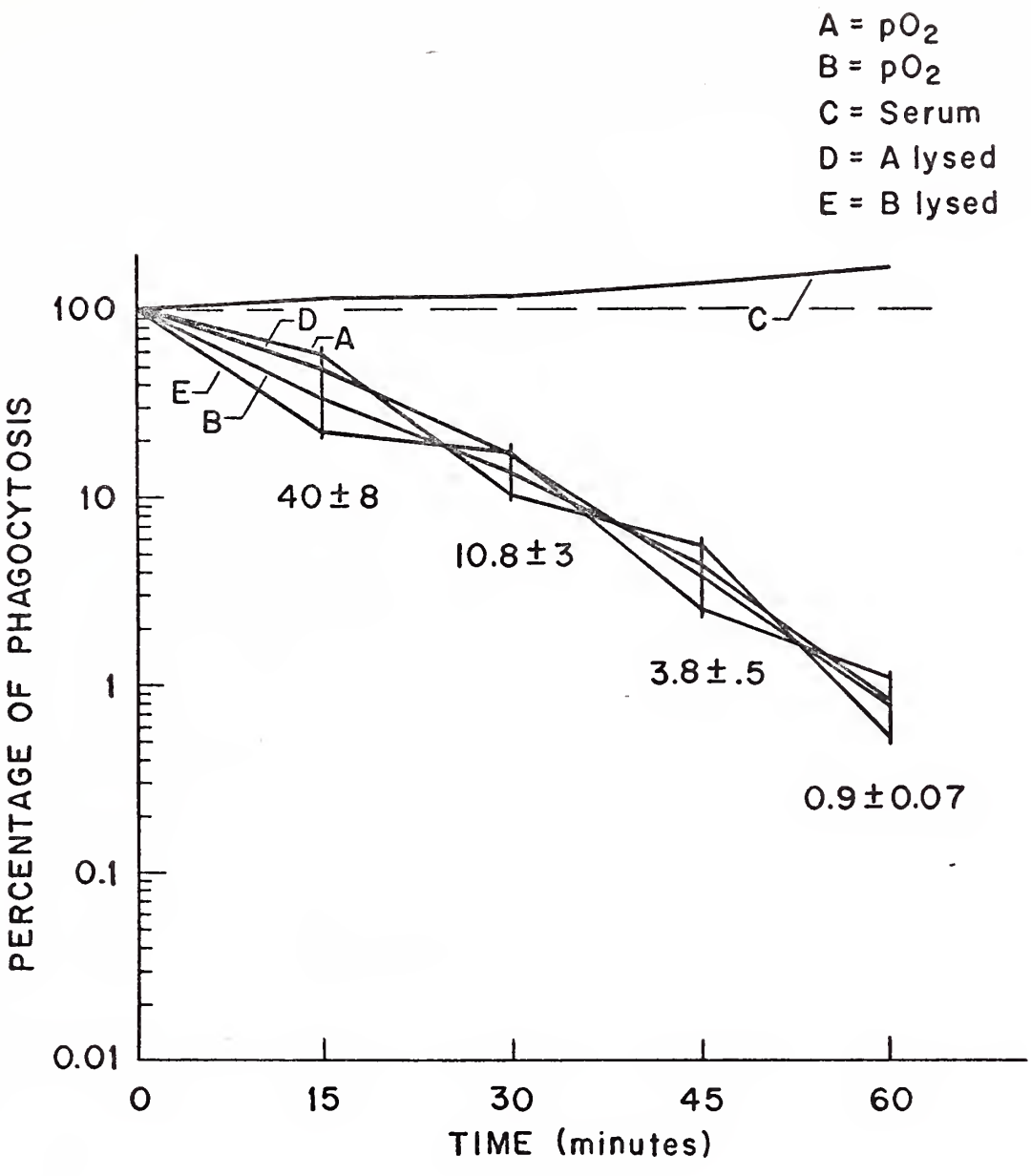
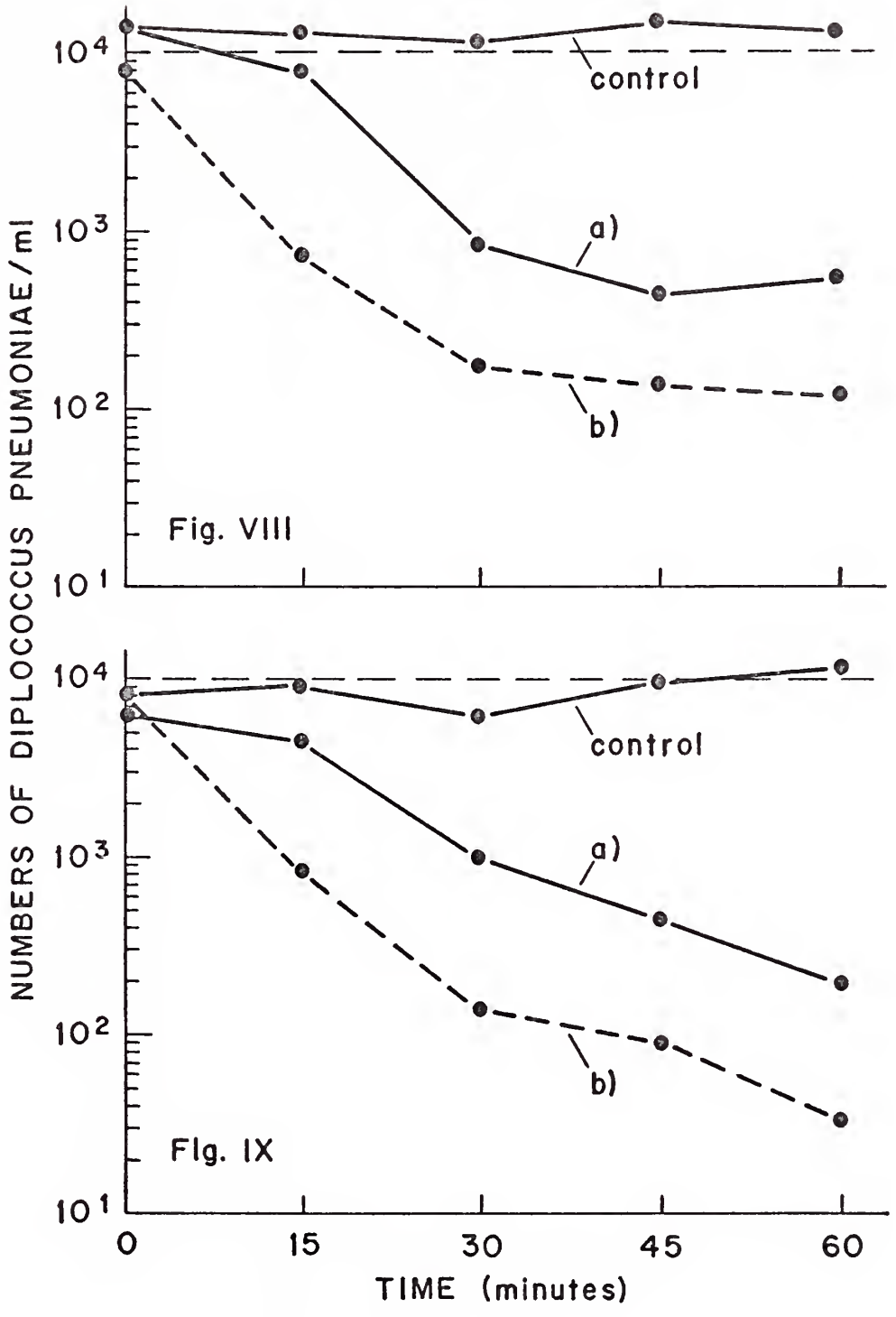


Figure VII: Blood phagocytic-bactericidal activity in sickle cell anemia. Mean and standard deviations for 8 patients (See Table V). The half life of viable pneumococci is comparable to that of control blood in spite of the greater number of WBC's (Average = 10.04×10^3) in these patients. This suggests a relative decrease in blood bactericidal activity either due to abnormal phagocytes or abnormal serum factors.

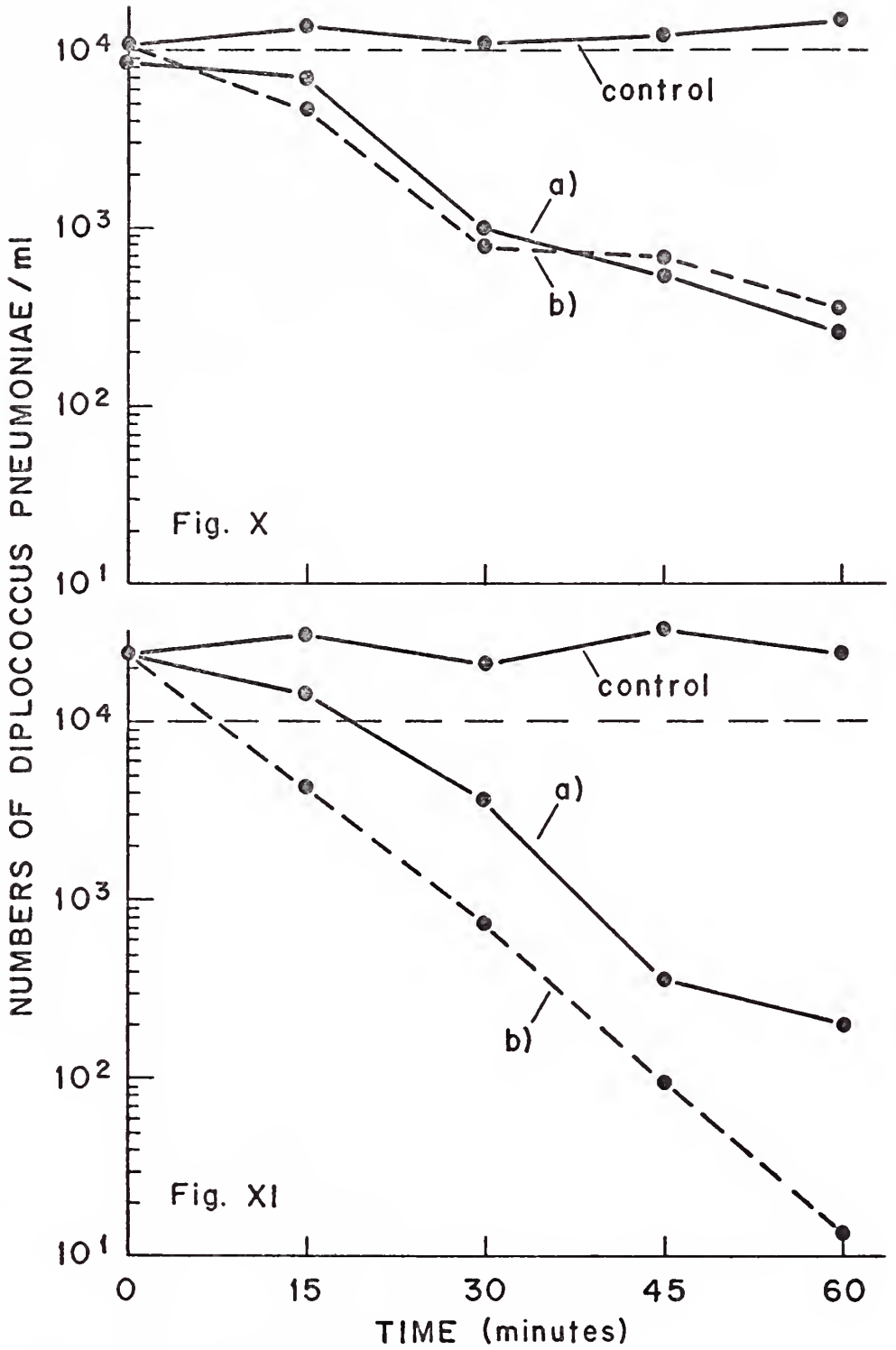
2. Since the patients with SS disease had higher white blood cell concentrations, it would have been expected that their phagocytic response be greater than normal. The relatively low rate of phagocytosis for these patients could be explained by either abnormal phagocytes or abnormal serum factors (opsonins). In order to demonstrate that serum factors are responsible for diminished phagocytic activity, it was necessary to repeat the above procedures with normal serum substituted for the serum of patients with Hgb SS. Great care was taken to match blood groups between patients and serum donors. Also, it was deemed necessary to maintain the original hematocrit of the patient, i.e. if the original Hct. was 20.0%, then 80 cc's of normal serum was eluted to every 20 cc's of the patient's packed red blood cells (Hgb SS). Six patients were chosen for part B, with the same scrutiny as those of part A (see Table VI).

TABLE VI

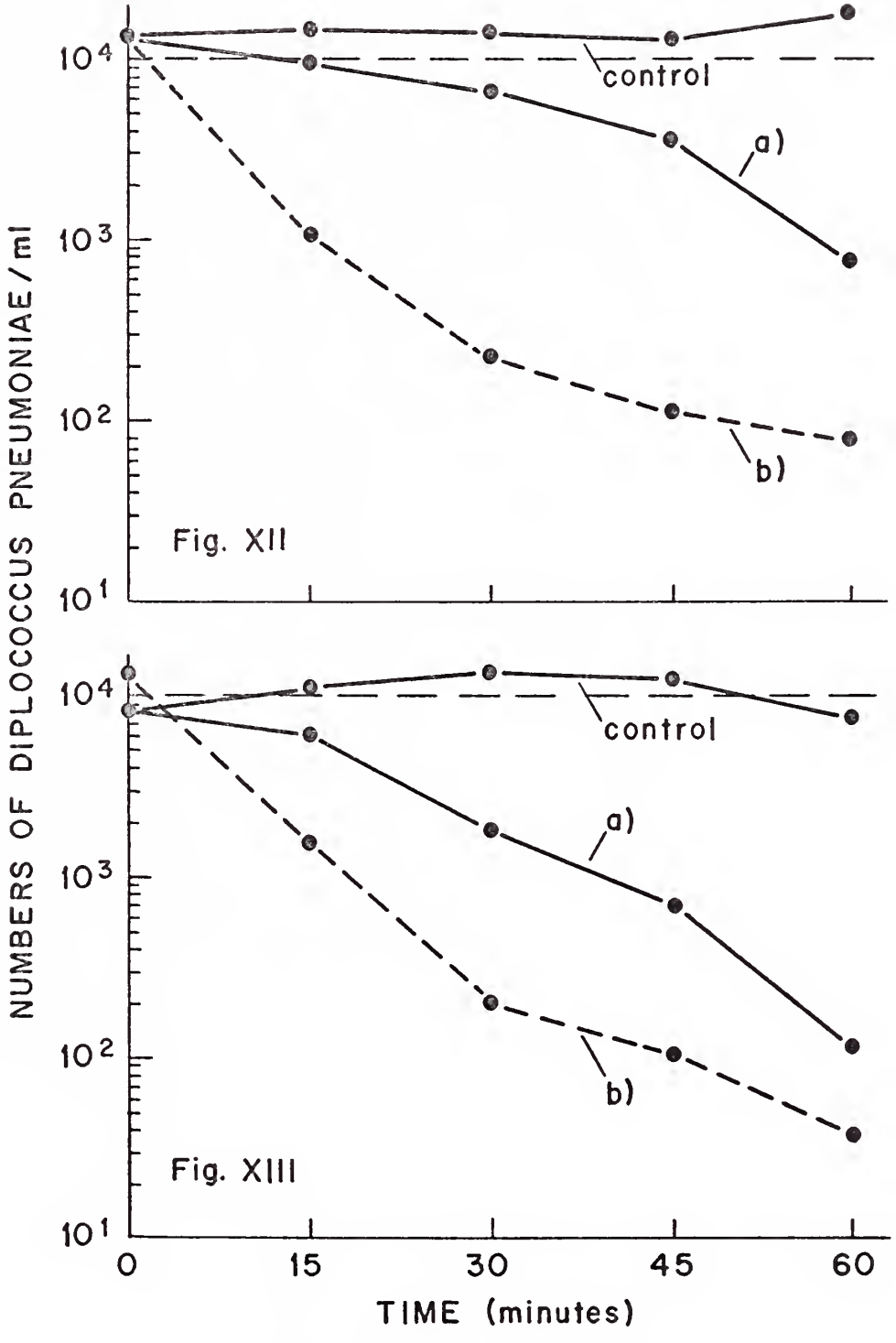
Pt.	Int.	Age	Sex	Hct.	Type	F	A_2	%S	WBCx10^3	Seg:Band
9	A.E.	15	F	24.0	SS	2.0	3.2	94.8	15.7	48:10
10	D.G.	19	F	17.0	SS	12.4	1.3	86.3	14.1	55:13
*11	K.Z.	6	F	25.0	SS	6.0	3.0	91.0	15.0	44:8
12	M.M.	12	F	18.0	SS	6.4	3.8	89.8	9.3	50:1
13	T.L.	11	M	13.9	SS	4.3	2.7	93.0	12.8	41:5
14	J.W.	15	M	16.0	SS	1.9	3.6	95.5	6.4	52:3



Figures VIII and IX: Demonstration of effect of substituting normal AA_2 serum (b) for SS serum (a) in patients #9 (A.E.) and #10 (D.G.), (c) = control.



Figures X and XI: Demonstration of effect of substituting normal AA_2 serum (b) for SS serum (a) in patients #11 (K.Z.) and #12 (M.M.). (c) = control.



Figures XII and XIII: Demonstration of effect of substituting normal AA_2 serum (b) for SS serum (a) in patients #13 (T.L.) and #14 (J.W.). (c) = control.

Figures VIII to XIII show that in 5 out of 6 patients the addition of serum from control subjects resulted in increased phagocytic activity for pneumococcus.

Figure XIV shows mean and standard deviation from the phagocytosis experiments using sickle cell blood with and without substitution, with control (AA) serum. Each patient experiment served as its own control, and all curves were integrated into one graph, with standards of deviations and percentages of phagocytosis per unit time. Phagocytic activity in patients with hemoglobin SS appears significantly more efficient with than without normal serum (see Figure XIV). To determine how clearly the experimentally determined sets of ratios differed at 30 and 60 minutes, a two sample T-test was performed at each of these time points[11,16,28]. At 30 minutes T was 11.60 with 8 degrees of freedom giving a p value of < 0.001. At 60 minutes T was 2.454 with 8 degrees of freedom giving a p value of < 0.05.

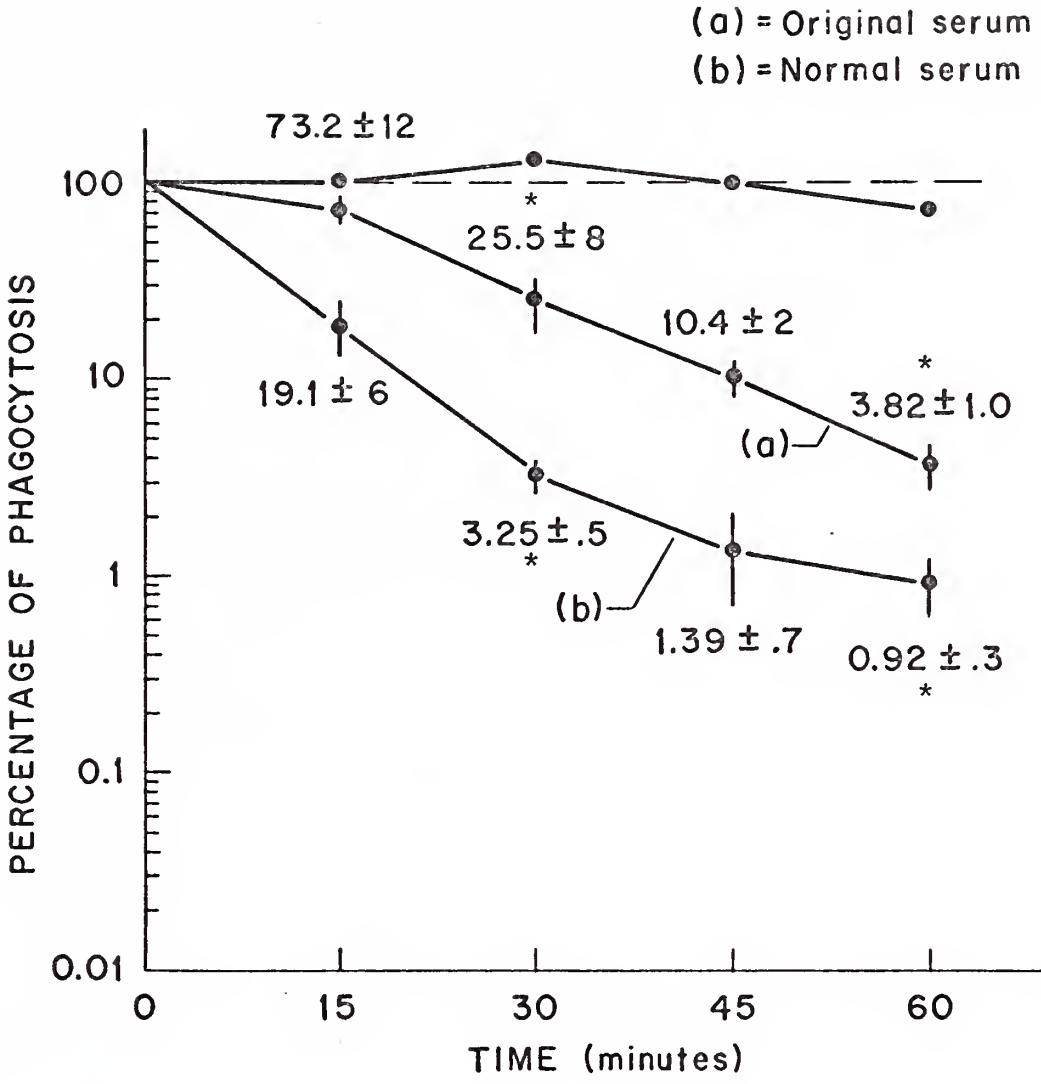


Figure XIV. Enhancement of blood phagocytic activity in the blood of SS individuals by substitution of control serum. (a) Mean and standard deviations of phagocytic-bactericidal study using original autologous serum. (b) Mean and standard deviations of phagocytic-bactericidal study after substitution of serum with blood group compatible control serum. * The difference between these points was statistically significant.

DISCUSSION

A number of investigators have pointed out an association between the hemolytic states of sickle cell disease and serious bacterial infections[12,22]. The clinical course of patients with sickle cell anemia is characterized by painful, intermittent, vaso-occlusive crises. Nevertheless, infections and not crises are the most common reason for hospitalization and the greatest cause of death in this patient population. Barrett-Connors showed that those patients with hematologic crises and bacteremia had a high mortality rate[3]. She noted the following general mechanism: The reticulo-endothelial system, i.e. liver, spleen and blood phagocytes, function to take up bacteria introduced into the blood stream. Once the phagocytic cells have ingested the organisms, antigens are passed along the lymphoid cells which recognize the antigen as foreign and initiates the production of specific antibody. Bactericidal activity of the serum against gram negative bacteria depends on lysozyme, complement and antibody. But the major killing of gram positive bacteria is intracellular and therefore dependent upon phagocytosis (and specific antibody, C' and other heat labile factors). While evidence for a defective integumentary system (first line of defense against

bacterial infections) is lacking in this patient population, evidence for an exhausted RES (second major line of defense against bacterial infections) secondary to excessive erythrophagocytosis, infarctions, etc., could explain the increased incidence of bacteremia.

In the young with Hgb SS, the spleen is large as a result of erythrophagocytosis and trapping of sickled cells. Later this organ is destroyed by infarcts and fibrosis. Pearson et al. reported a state of impaired splenic reticeloendothelial activity ("functional asplenia") in children with Hgb SS[25].

Other investigators have recently described deficiencies involving serum factors that specifically opsonize the bacterial cell wall. The absence or diminution of adequate opsonization results in the failure of the phagocytic cell to engulf the infecting or target organism. The clearest examples of these are deficiencies in certain components of the complement. There are other less defined factors which nevertheless act on the bacterial cell. In all instances, when the missing opsonizing or chemotactic factor is replaced, the ability of the phagocyte to engulf the particle is completely regained[2,17].

A new syndrome[7] has been described in which there is a clear deficiency in a newly recognized factor which, by contrast to the aforementioned factors, acts directly on

on the phagocytic cell and not on the target particle. It is a tetrapeptide that is made in the spleen and exhibits all the properties that characterize peptide hormones. It is called Tuftsin, after Tufts University[19]. Interestingly, splenectomized human subjects show no phagocytic stimulation and no inhibitory effect of tuftsin activity. Investigators have failed to isolate from such patients a peptide with the physicochemical cheracteristics of the normal peptide. Having previously shown that in splenectomized dogs tuftsin activity also disappears[18], it would appear that tuftsin is at least inserted on the carrier leukokinin molecule in the spleen, and is probably synthesized there also. Each patient with a deficiency of its phagocytosis stimulating properties has a history of recurrent infections. The deficiency in these patients was shown to be due probably to a structural defect on the molecule (L-threonyl-L-lysysyl-L-prolyl-L-Arginine) rather than in complete absence of the tetrapeptide -- as is most likely the case in splenectomized patients. Tuftsin may be deficient in adults with sickle cell anemia because they are auto-splenectomized.

A factor has been isolated from normal serum which is necessary for phagocytosis of pneumococci and was found deficient in the serum of children with sickle cell

disease. It behaved like heat-labile opsonin since its primary effect was upon the bacteria, rendering them more susceptible to infection by phagocytosis. (In the preparation of tuftsin it is incubated at 37° C for 30 minutes ... and placed in a water bath at 80° C for 8 minutes ...). The serum opsonizing activity for the pneumococcus was studied with a phagocytic test using leukocyte-HBG suspensions and stored serum from normals and patients with sickle cell disease. Heat-labile serum opsonizing activity for the pneumococcus was markedly deficient in serum from the patients, with a mean phagocytosis of 6.5% whereas control children with AA_2 hemoglobin showed a mean value of 35.1%. Serum opsonizing activity for salmonella was comparable in patients with sickle cell disease and controls, although much of the activity was heat stable. Hemolytic complement activity was similar in both groups of children[29]. Since pathologic changes in the spleen in sickle cell disease may result in functional autosplenectomy, these observations may reflect the impairment of splenic clearance and heat-labile opsonin synthesis.

Because of the newly apparent incidence of infections with diplococcus pneumoniae in children with sickle cell anemia[26], these several investigators agree: (1) the reticulo-endothelial system is important in clearance of

these infections and (2) this system is heavily dependent upon a serum factor --- opsonin[4]. In the normal leukocytic response to bacterial infection, phagocytosis is the rate limiting step[6] and most ingested bacterial species are killed rapidly following the process[30]. A defect that hinders, efficient, rapid phagocytosis could permit rapid multiplication and dissemination of a small bacterial inoculum within the blood stream, analogous to what occurs in vitro in the culture broth.

The relevance of diminished serum opsonizing activity to acute pneumococcal disease can be examined in the light of a number of considerations. A syndrome of fulminant pneumococcal septisemia and meningitis has been documented in the young hyposplenic[15] and autosplenectomized child[9]. This same predisposition to overwhelming infection is shared by infants and youth with Hgb SS[14]. When antibody levels are low or absent, clearance of ^{125}I-labelled pneumococci occurs preferentially through the spleen[24]. Consequently the spleen is the first line of defense against blood borne particulate material like pneumococcus. The spleen is also responsible for elaborating some type of early antibody response to antigenic stimulation[25]. The dual role of this extension of the reticulo-endothelial system appears to be: (1) phagocytosis and (2) clearance of early antibody synthesis.

If the spleen is responsible for the synthesis of opsonin, the pathological changes which occur therein, in patients with Hgb SS, may explain the findings of low pneumococcal opsonic activity in this patient population. Progressive vascular occlusion and infarction[8] of the spleen of infants, results in those organs being shrunken and fibrotic in older patients with Hgb SS. The higher incidence of splenomegaly in young children with Hgb SS and pneumococcal sepsis is attributable to "functional asplenia"[21]. This organ removes encapsulated organisms, i.e. pneumococcus, in the presence of normal splenic function.

In the presently conducted experiments, when whole blood from normals was compared with whole blood of patients with Hgb SS under similar conditions of oxygen tension, temperature and osmotic lysis, there was no difference in the phagocytic-bactericidal rates. Due to the high leukocyte/bacterial ratio used throughout all phases of these tests, cell-associated clumping was not an artifact[5]. When the serum of patients with Hgb SS was replaced with fresh normal serum, there was a statistically significant increase in phagocytosis. The data reported here is highly suggestive of a humoral deficiency[10] in this patient population. It should be made clear that the assay system employed herein has

measured the effect of this humoral deficiency and not the concentration of the serum factor involved.

Only log phase organisms were used throughout this experiment (quick frozen, post 4 hours growth, at 37° C) since older ones lose virulence by virtue or loss of capsular polysaccharide. The half life of pneumococcus in this in vitro test system was 12.75 minutes, an extremely short doubling time -- thus providing further credibility to the extensive list of documented fulminant infections in this patient population. As a result of enhanced phagocytic response rates in patients with substituted normal serum in 83.3% of the population studied, this data raises the possibility that therapy with normal plasma may decrease the mortality of the infectious complications of sickle cell anemia and related hemoglobinopathies.

SUMMARY

Phagocytosis of pneumococci by blood leukocytes in a group of 14 patients with sickle cell hemoglobinopathies was compared to that of a control of 6 subjects with Hgb AA_2. An in vitro test system was used in which phagocytosis was quantitated by the number of pneumococcal organisms killed after incubation with control versus

Hgb SS blood.

1. The rate of phagocytosis of pneumococci was impaired in the whole blood of patients with Hgb SS compared to normals.

2. The growth rates of pneumococcus was not altered by:
   (a) Use of Hgb SS blood versus Hgb AA_2 blood
   (b) Use of low 0_2 tension versus room air.

3. The rate of phagocytosis in Hgb SS blood was increased when control serum was used as a source of opsonin.

The increased susceptibility to pneumococcal infection in patients with Hgb SS may be related to the decreased opsonic capacity of their blood. Functional asplenia may account for this humoral deficiency which apparently may be replenished in this patient population by adding normal human plasma.

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